JOURNAL OF INTERFERON & CYTOKINE RESEARCH
Volume 31, Number 2, 2011

DOI: 10.1089/jir.2010.0055

Porcine Type I Interferon Rapidly Protects Swine Against Challenge with Multiple Serotypes of Foot-and-Mouth Disease Virus

Camila C.A. Dias,[1,2] Mauro P. Moraes,[1] Fayna Diaz-San Segundo,[1,2] Teresa de los Santos,[1] and Marvin J. Grubman[1]

Foot-and-mouth disease virus (FMDV) causes a highly contagious disease of cloven-hoofed animals. Current inactivated vaccines require approximately 7 days to induce protection, but before this time vaccinated animals remain susceptible to disease. Previously, we demonstrated that intramuscular (IM) inoculation of a replication-defective human adenovirus type 5 (Ad5) vector containing a porcine interferon α gene (*pIFNα*) can protect swine challenged 1 day later by intradermal (ID) injection with FMDV A24 Cruzeiro from both clinical disease and virus replication. To extend these studies to other FMDV serotypes, we demonstrated the effectiveness of Ad5-pIFNα against ID challenge with O1 Manisa and Asia-1 and against A24 Cruzeiro in a direct contact challenge model. We also showed that an Ad5 vector containing the *pIFNβ* gene can protect swine against ID challenge with A24 Cruzeiro. Further, IM inoculation of a 10-fold lower dose of Ad5-pIFNα at 4 sites in the neck compared with 1 site in the hind limb can protect swine against ID challenge. These studies demonstrate the ability of Ad5-delivered type I IFN to rapidly protect swine against several FMDV serotypes and suggest that various modifications of this approach may enable this strategy to be successfully used in other FMD susceptible species.

Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals that has significant economic consequences in affected countries. The infectious agent, FMD virus (FMDV), is a member of the *Aphthovirus* genus of the Picornaviridae family, and contains a single-stranded positive-sense RNA genome of about 8,500 nucleotides encapsidated by 60 copies each of 4 structural proteins (Grubman and Baxt 2004). FMDV is an antigenically variable virus consisting of 7 serotypes (A, O, C, Asia-1, and South African territories 1–3) and multiple subtypes (Domingo and others 2003). Currently, the disease is controlled by restriction of animal movement, slaughter of infected and in-contact susceptible animals, and possibly vaccination with an inactivated whole virus vaccine (Grubman and Baxt 2004). Administration of this vaccine or an experimental vaccine based on a replication-defective human adenovirus type 5 (Ad5) vector containing the FMDV capsid and 3C proteinase coding regions requires approximately 7 days to induce protective immunity in animals (Moraes and others 2002; Golde and others 2005; Pacheco and others 2005). However, since FMDV infection results in rapid replication and spread within the host and shedding of virus into the environment, animals exposed to virus before 7 days postvaccination are still susceptible to the disease. As a result we have initiated a program to stimulate a rapid innate response to protect animals before vaccine-induced adaptive immunity. We anticipate that this approach would be used in combination with vaccination to induce both a rapid and specific long-lasting protective immune response (Grubman 2003, 2005; Moraes and others 2003; de Avila Botton and others 2006).

Type I interferon (IFNα/β) is the first line of host defense against viral infection and upon its induction and secretion it causes upregulation of hundreds of IFN-stimulated genes (ISGs) and their products (Der and others 1998; Takaoka and Yanai 2006; Fontana and others 2008). We and others have shown that replication of all FMDV serotypes is inhibited in cell culture by pretreatment with type I IFN (Ahl and Rump 1976; Chinsangaram and others 1999, 2001; Moraes and others 2007). More recently, we constructed an Ad5 vector containing porcine IFNα (Ad5-pIFNα) and demonstrated

[1]Plum Island Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Greenport, New York.
[2]Plum Island Animal Disease Center Research Participation Program, Oak Ridge Institute for Science and Education, Oak Ridge, Tennessee.

that swine inoculated with this vector intramuscularly (IM) at 1 site in the hind limb produce significant levels of pIFNα and are completely protected when challenged by intradermal (ID) inoculation with FMDV serotype A24 Cruzeiro 1 day later (Chinsangaram and others 2003). Further, protection lasts for 3–5 days and even treatment 1 day postchallenge reduces viremia and clinical disease (Moraes and others 2003). In preliminary studies we have demonstrated that Ad5-pIFNα can enhance the efficacy of our Ad5-FMD vaccine, indicating that IFN does not appear to adversely affect the adaptive immune response (de Avila Botton and others 2006). We have also initiated studies to understand the molecular mechanisms induced by IFN treatment that result in protection against FMDV challenge and found a correlation between protection and both, specific ISG upregulation and tissue specific infiltration of dendritic cells (DCs) and natural killer (NK) cells (Moraes and others 2007; Diaz-San Segundo and others 2010).

Although these proof-of-concept studies demonstrated that Ad5-pIFNα can rapidly protect swine against FMDV, there are limitations to its utility, including the following: (1) only 1 FMDV serotype has been tested, (2) treatment requires relatively high doses to induce protection in swine, (3) protection against the natural route of FMDV infection has not been examined, and (4) treatment of bovines only results in delay of disease onset and severity (Wu and others 2003). To address some of these limitations, in this study we have tested the efficacy of this approach against other FMDV serotypes, including O1 Manisa and Asia-1; examined its efficacy in a direct contact challenge model, which is the natural route of FMDV infection; and examined alternative routes of administration of the Ad5 vector. Further, we also evaluated the efficacy of an Ad5 vector containing the gene for pIFNβ (Adt-pIFNβ) against ID challenge with A24 Cruzeiro.

Our results indicate that Ad5-pIFNα, at a dose of 10^{11} focus forming units (FFU)/animal, is able to completely protect swine against all 3 serotypes of FMDV, while treatment with a 10-fold lower dose results in complete protection of some animals and delay in disease onset and severity and reduction in virus shedding in the remaining treated animals. Further, Ad5-pIFNα-inoculated animals were also protected when challenged with FMDV A24 Cruzeiro by direct-contact exposure. Pretreatment with a 10^{11} FFU/animal dose of Adt-pIFNβ also completely protected swine against ID challenge with A24 Cruzeiro. Notably, we were able to reduce the protective dose of Ad5-pIFNα 10-fold when swine were inoculated IM at 4 separate sites in the neck. These results demonstrate the utility of this approach in conferring rapid protection in swine after either ID or direct contact challenge with FMDV and suggest that various modifications of this strategy can overcome some of its current practical limitations.

Materials and Methods

Cells and viruses

Baby hamster kidney cells (BHK-21, clone 13) were used to measure FMDV titers in plaque assays, and a swine kidney cell line (IBRS-2) was used to measure antiviral activity in plasma from inoculated animals by a plaque reduction assay (Chinsangaram and others 2001). FMDV serotypes A24 Cruzeiro, O1 Manisa, and Asia-1 were obtained from the vesicular fluid of infected swine, titered in both swine (see below) and in tissue culture, and stored in aliquots at −70°C.

The replication-defective human Ad5 vectors containing the *pIFNα* or *pIFNβ* genes, constructed as described by Gall and others (2007), and the control vectors AdNull and Ad-Luciferase were obtained from GenVec, Inc., through an agreement with the Department of Homeland Security, Office of Science and Technology. The pIFN genes were supplied by our lab to GenVec, Inc., for use in the construction of Ad5 vectors containing type I IFNs (designated Adt-pIFNα and Adt-pIFNβ). All experiments were performed with the same set of vector lots.

Animal studies

All animal experiments were performed under a protocol reviewed and approved by the Institutional Animal Care and Use Committee of the Plum Island Animal Disease Center. Yorkshire pigs weighing about 35–40 lbs each were used in all experiments and acclimated for approximately 4–5 days before the start of the experiments. To determine the challenge dose of each FMDV serotype to use in ID inoculation studies, groups of 4 swine were infected ID in the hind heel bulb using 4 sites of inoculation, 100 μL per site, with low (10^4 tissue culture infectious dose $[TCID]_{50}$/animal), medium (10^5 $TCID_{50}$/animal), or high (10^6 $TCID_{50}$/animal) doses of FMDV A24 Cruzeiro, O1 Manisa and Asia-1 in separate experiments (Supplemental Table S1, available online at www.liebertonline.com). On the basis of these studies we selected a challenge dose of 10^5 $TCID_{50}$ for all serotypes, which is 10-fold higher than the challenge dose recommended by the World Organization of Animal Health (OIE 2004).

In the IFN titration studies, groups of 3 swine were inoculated with 3 different vector doses (10^9 FFU/animal, 10^{10} FFU, and 10^{11} FFU) of Adt-pIFNα or Adt-pIFNβ to evaluate the biological activity of those constructs. Swine inoculated with AdLuciferase or AdNull (10^{11} FFU/animal) were used as controls. Animals were inoculated IM in 1 site in the right hind limb with 2 mL of the respective vector and subsequently monitored for 4 days for possible adverse signs as a result of vector administration and blood assayed for IFN expression and antiviral biological activity.

In the subsequent efficacy studies animals were inoculated IM with 2 mL or 3 mL vector in 1 site in the right hind limb or neck or with 0.50 or 0.75 mL vector per site in 4 sites, 2 in each hind limb or 2 in both sides of the neck. All Adt-pIFNα/β-inoculated animals were housed in double-gated rooms (2 animals per room) so that they had no direct contact, except in the initial A24 Cruzeiro ID challenge experiment, in which all animals in a group were housed in the same room.

A direct contact challenge experiment was performed based on an experimental protocol developed by Pacheco and others (manuscript in preparation). Six donor animals were ID inoculated in the heel bulb with FMDV A24 Cruzeiro. When vesicular lesions were apparent, approximately 2 days postchallenge (dpc), the 6 donor animals were comingled with Adt-pIFNα-treated and control animals in a ratio of 1:2 for 18 h. After exposure, donor animals were euthanized, and the Adt-pIFNα-treated and control animals were relocated to double-gated rooms (2 animals per room).

In all challenge experiments, animals were monitored daily for 10 days for clinical signs, including fever, alertness,

lameness, and development of vesicles on the coronary band of the hooves, on the snout and mouth. Lesion scores of the animals were determined by the number of digits plus snout and mouth with vesicles (maximum score is 17).

Blood and nasal swab sampling

Blood samples were drawn from the anterior vena cava at the times indicated in each experiment. Serum was obtained from blood drawn into nonheparinized tubes and tested for viremia and neutralizing antibodies using a standard plaque reduction assay as described below. Plasma was obtained from the blood drawn into heparinized tubes and tested to determine levels of antiviral biological activity of pIFNα/β as described in the following section and pIFNα protein by ELISA. Nasal swabs were collected starting the day of challenge and for the following 6 days and tested for the presence of FMDV by titration in BHK-21 cells.

Detection of FMDV RNA by real-time reverse transcriptase–polymerase chain reaction

One to 7 dpc frozen serum samples from animals that had no detectable clinical disease were thawed and processed for RNA extraction and real-time reverse transcriptase–polymerase chain reaction (rRT-PCR) as previously described (Pacheco and others 2010). Samples were considered positive when Ct values were <40.

Interferon biological assays

Antiviral activity was evaluated in plasma samples as previously described (Moraes and others 2003; de Avila Botton and others 2006). In brief, samples were obtained at 0–4 days postinoculation (dpi), diluted, and incubated on IBRS-2 cells; after 24 h supernatants were removed, and the cells infected for 1 h with approximately 100 plaque forming units (PFU) of FMDV serotype A12 and overlaid with gum tragacanth. Plaques were observed 24 h later by staining with crystal violet. Antiviral activity (U/mL) was reported as the reciprocal of the highest supernatant dilution that resulted in a 50% reduction in the number of plaques relative to the number of plaques in the mock-treated infected cells.

Interferon-α ELISA

ELISA was performed as previously described (Moraes, and others 2003). Porcine IFNα concentrations were expressed in picograms per milliliter and calculated by linear regression analysis of a standard curve generated with serial 2-fold dilutions of recombinant pIFNα (PBL Biomedical Laboratories). All samples were assayed in duplicate. Levels of pIFNα protein of <200 pg/mL were not considered meaningful.

Plaque reduction neutralization (PRN_{70}) assay

Sera samples were collected at 0, 7, 14 and 21 dpc for each experiment and heated at 56°C for 30 min, and aliquots stored at −70°C. Sera were tested for the presence of neutralizing antibodies against FMDV in a PRN assay (Mason and others 1997). Neutralizing titers were reported as the serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}). A titer of 128 or higher was considered to indicate productive virus replication.

3ABC ELISA assay

Swine sera from 0 and 21 dpc were examined for the presence of antibodies against FMDV nonstructural (NS) protein 3ABC using a PrioCHECK™ FMDV-NS ELISA kit (Prionics AG) following the manufacturer's instructions (Sorensen and others 1998).

Results

Antiviral response in swine inoculated with Adt-pIFNα or Adt-pIFNβ

To determine the doses of Adt-pIFNα and Adt-pIFNβ necessary to produce levels of antiviral activity previously found sufficient to partially or completely protect swine against ID challenge with FMDV A24 Cruzeiro (Chinsangaram and others 2003; Moraes and others 2003), we performed a dose–response potency study. Groups of 3 swine were inoculated IM at 1 site in the right hind limb with low (1×10^9 FFU), medium (1×10^{10} FFU), or high doses (1×10^{11} FFU) of Adt-pIFNα or Adt-IFNβ, and 1×10^{11} FFU of Ad-Luciferase control vector, in 2 separate experiments. Biological activity and levels of pIFNα were assayed in plasma samples at the time of inoculation and for 3 additional days (Table 1). We were unable to determine the levels of pIFNβ protein since the appropriate reagents for this cytokine are not currently available. The average biological activity in plasma samples from animals in the high-dose Adt-pIFNα-inoculated group was 1,333 U/mL at 24 h after inoculation and activity was detectable for 1 additional day (Table 1). The average pIFNα protein at 1 dpi in the high-dose group was 26,977 pg/mL, and this protein was detectable for 2 additional days. In the medium-dose group biological activity was 92 U/mL at 1 dpi and was detectable for an additional day, whereas pIFNα protein (1,184 pg/mL) was only detectable for 1 day. No antiviral activity or pIFNα protein was detected in either the low-dose IFN group or in the control group.

In the Adt-pIFNβ dose–response experiment the average biological activity in plasma samples from animals in the high-dose-inoculated group was 667 U/mL at 1 dpi and was detectable for a second day, whereas biological activity was only detectable for 1 day in the medium-dose group (Table 1). A low level of pIFNα protein was only detectable for 1 day in the high-dose-inoculated group. The animals inoculated with the low-dose pIFNβ or the control vector did not develop either detectable antiviral activity or pIFNα protein. On the basis of these results we selected the medium and high doses of Adt-pIFNα and Adt-pIFNβ to test in efficacy studies.

Clinical response of swine pretreated with Adt-pIFNα and challenged by ID inoculation with FMDV A24 or O1 Manisa

Groups of 3 animals were administered medium (1×10^{10} FFU) or high doses (1×10^{11} FFU) of Adt-pIFNα or a high dose of AdLuciferase or AdNull and challenged 24 h later with 10^5 $TCID_{50}$ of either FMDV A24 or O1 Manisa. In the A24 challenge experiment all the control-inoculated animals developed viremia and clinical disease by 2 dpc and none had detectable antiviral activity or pIFNα protein (Table 2). In the Adt-pIFNα medium-dose group all the animals

Table 1. Dose Response of Swine Inoculated with Adenovirus Type 5 Vector Containing a Porcine Interferon α or β Gene

		Antiviral activity (U/mL)[b]					*pIFNα (pg/mL)*[c]				
Group (dose FFU)[a]	*Animal no.*	*0 dpi*	*1 dpi*	*2 dpi*	*3 dpi*	*4 dpi*	*0 dpi*	*1 dpi*	*2 dpi*	*3 dpi*	*4 dpi*
AdLuciferase (1×10^{11})	06	<25	<25	<25	<25	<25	0	0	0	0	0
	07	<25	<25	<25	<25	<25	0	0	0	0	0
	08	<25	<25	<25	<25	<25	0	0	0	0	0
Adt-pIFNα (1×10^{9})	09	<25	<25	<25	<25	<25	0	0	0	0	0
	10	<25	<25	<25	<25	<25	0	137	0	0	0
	11	<25	<25	<25	<25	<25	0	74	0	0	0
Adt-pIFNα (1×10^{10})	12	<25	25	<25	<25	<25	0	197	0	0	0
	13	<25	50	25	<25	<25	0	820	66	0	0
	14	<25	200	50	<25	<25	0	2,536	380	0	0
Adt-pIFNα (1×10^{11})	15	<25	800	400	25	<25	0	27,688	4,470	239	0
	16	<25	1,600	800	50	<25	0	13,424	6,246	754	30
	17	<25	1,600	400	25	<25	0	39,818	5,495	783	43
AdLuciferase (1×10^{11})	26	<25	<25	<25	<25	<25	0	0	0	0	0
	27	<25	<25	<25	<25	<25	0	0	0	0	0
	28	<25	<25	<25	<25	<25	0	0	0	0	0
Adt-pIFNβ (1×10^{9})	29	<25	<25	<25	<25	<25	0	0	0	0	0
	30	<25	<25	<25	<25	<25	0	0	0	0	0
	31	<25	<25	<25	<25	<25	0	0	4	0	0
Adt-pIFNβ (1×10^{10})	32	<25	<25	<25	<25	<25	0	0	0	0	0
	33	<25	50	25	<25	<25	0	190	39	0	0
	34	<25	<25	<25	<25	<25	0	0	232	0	0
Adt-pIFNβ (1×10^{11})	35	<25	800	400	50	25	0	250	91	0	0
	36	<25	800	100	<25	<25	0	316	100	0	0
	37	<25	400	200	<25	<25	0	139	15	47	0

[a]Dose of inoculum per animal expressed as number of FFU in 2 mL of phosphate buffered saline (PBS).
[b]Highest dilution that reduces foot-and-mouth disease virus A12 plaque number by 50%.
[c]Amount of pIFNα in plasma samples determined by ELISA.
Adt-pIFNα, adenovirus type 5 vector containing a porcine interferon α gene; dpi, days postinoculation; FFU, focus forming units.

developed low levels of pIFNα protein and antiviral activity on day 1, which lasted for 1 additional day (data not shown). One animal in this group developed very low levels of viremia and clinical disease at 4 dpc (no. 20961), whereas the other 2 animals developed viremia by 6 or 7 dpc and lesions at 7 and 10 dpc, respectively. All 3 animals in the Adt-pIFNα high-dose group had ~14–21,000 pg/mL pIFNα protein and ~1,300–2,300 U/mL antiviral activity 1 day post-administration, and this continued at reduced levels for an additional 2–3 days (data not shown). None of the animals in this group developed clinical disease, viremia, or virus in nasal secretions, and they were all 3ABC and rRT-PCR negative (Tables 2 and 7). However, 2 of the animals, nos. 20962 and 20964, had significant levels of FMDV-specific neutralizing antibodies.

In this initial efficacy study each group of Adt-pIFNα-treated animals was kept in separate rooms and had direct intragroup contact throughout the experiment. Since 1 animal in the medium-dose group, no. 20961, developed lesions at 4 dpc, while the other 2 animals, nos. 20959 and 20960, only developed lesions 3–6 days later, it is possible that the later 2 animals developed FMD because of long-term direct exposure to animal no. 20961 at a time when the protective effects of pIFNα had waned. As a result in all subsequent studies the Adt-pIFNα/β groups were kept 2 animals/room with a double gate separating each animal to prevent direct contact.

In the FMDV O1 Manisa challenge experiment all the control animals developed clinical disease by 1–3 dpc, but only 2 animals in this group had detectable, but low levels of viremia (Table 2). One animal in the group treated with 10^{10} FFU Adt-pIFNα, no. 960, did not develop antiviral activity or meaningful levels of pIFNα and had clinical disease at 3 dpc. Another animal in this group developed lesions by 4 dpc, but disease severity was considerably milder than in the control animals. The other animal, no. 959, was completely protected from clinical disease, had no viremia or virus in nasal swabs, was 3ABC and rRT-PCR negative, and had no detectable FMDV-specific neutralizing antibodies (Tables 2 and 7). All 3 animals in the 10^{11} FFU group had high levels of pIFNα protein and antiviral activity. None of the animals developed clinical disease or viremia, or shed virus, and they were all 3ABC and rRT-PCR negative and had no detectable FMDV-specific neutralizing antibodies (Tables 2 and 7).

Clinical response of swine pretreated with Adt-pIFNα and challenged with FMDV A24 Cruzeiro by contact

Twelve naive animals were inoculated IM with either 1×10^{10} or 1×10^{11} FFU Adt-pIFNα, 1×10^{11} FFU AdNull, or phosphate buffered saline (PBS). Twenty-four hours later they were brought into the same room with donor animals that had been infected 2 days earlier with FMDV and showed clear signs of disease. After 18 h of comingling, the donors were euthanized, whereas the other animals were distributed, 2 animals per room, separated by a double gate and monitored for 10 days. Virus was detected in nasal swab samples from all animals at 24 h postcontact exposure, demonstrating that they were effectively exposed (data not shown).

Table 2. Serological and Clinical Response of Swine Pretreated with Adenovirus Type 5 Vector Containing a Porcine Interferon α Gene and Intradermal Challenged with Foot-and-Mouth Disease Virus A24 Cruzeiro or O1 Manisa

Group (dose FFU)[a]	Animal no.	Foot-and-mouth disease virus	Antiviral act./pIFNα[b]	Viremia[c]	Shedding virus[d]	Clinical score[e]	PRN_{70}[f]	3ABC ELISA[g]
AdLuciferase[h] (10^{11})	20956	A24	<25/180	$2/1.0\times10^{4}$	$2/1.5\times10^{3}/4$	2/14	1,600	P
	20957		<25/95	$1/8.5\times10^{3}$	$2/3.9\times10^{2}/4$	2/17	3,200	P
	20958		<25/147	$1/2.0\times10^{5}$	$2/2.0\times10^{2}/4$	2/17	12,800	P
Adt-pIFNα[h] (10^{10})	20959	A24	303/3,307	$6/2.1\times10^{4}$	$5/7.0\times10^{0}/2$	10/12	6,400	N
	20960		447/5,615	$7/1.4\times10^{4}$	$5/2.5\times10^{2}/2$	7/12	6,400	P
	20961		226/1,921	$4/3.4\times10^{1}$	$5/2.1\times10^{4}/3$	4/13	6,400	P
Adt-pIFNα[h] (10^{11})	20962	A24	2,011/20,917	0	0	0/0	512	N
	20963		1,305/14,215	0	0	0/0	<8	N
	20964		2,273/20,053	0	0	0/0	512	N
AdNull[i] (10^{11})	955	O1M	<25/183	$1/4.0\times10^{3}/2$	$2/5\times10^{1}/1$	2/17	2,048	P
	956		<25/7	0	0	3/15	256	P
	957		<25/63	$2/5.4\times10^{2}/1$	$3/1.1\times10^{2}/1$	1/17	256	P
Adt-pIFNα[i] (10^{10})	958	O1M	400/626	0	0	4/4	1,024	P
	959		400/1,015	0	0	0/0	8	N
	960		<25/95	0	0	3/17	256	P
Adt-pIFNα[i] (10^{11})	961	O1M	1,600/46,103	0	0	0/0	<8	N
	962		1,600/16,303	0	0	0/0	<8	N
	963		1,600/35,855	0	0	0/0	<8	N

[a]Dose of inoculum per animal expressed as number of FFU in 2 mL of PBS.
[b]Antiviral activity (U/mL) and pIFNα (pg/mL) at 1 day postinoculation.
[c]First day postchallenge (dpc) that viremia was detected, maximum amount of viremia in PFU/mL detected in sera samples, and the duration (days) of viremia.
[d]First dpc that shedding virus was detected, maximum amount of shedding virus in PFU/mL detected in nasal swab samples, and the duration (days) of shedding.
[e]dpc first signs of lesions/highest lesion score.
[f]Neutralizing antibody response reported as serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}) at 21 dpc.
[g]Detection of NS proteins in 21 dpc serum samples by 3ABC ELISA; N, negative; P, positive.
[h]In this experiment all 3 animals in each group were housed in the same room and were in direct contact throughout the experiment.
[i]In this experiment the animals administered IFN were housed 2 per room with a double gate separating them so that they had no direct contact.
PRN, plaque reduction neutralization; PFU, plaque forming units.

Four of the 6 animals in the control groups developed viremia by 3–4 days postcontact and by 3–5 days all of the viremic animals had clinical disease (Table 3). One control animal developed a lesion at 8 days postcontact, whereas the remaining control animal never developed clinical disease. All 5 control animals that had clinical disease as well as the control animal with no clinical disease developed significant levels of FMDV-specific neutralizing antibodies.

All animals inoculated with 1×10^{10} FFU Adt-pIFNα had detectable levels of pIFNα and antiviral activity 1 day after administration. However, 1 animal in this group, no. 9135, only had antiviral activity and pIFNα for 1 additional day and developed 1 lesion at 6 days postcontact challenge. The other 2 animals had detectable antiviral activity/pIFNα for 2–3 additional days (data not shown); had no clinical disease, viremia, or virus in nasal swabs; were 3ABC and rRT-PCR negative; and had no detectable FMDV-specific neutralizing antibodies (Tables 3 and 7).

All animals inoculated with the high dose of Adt-pIFNα had significant levels of pIFNα and antiviral activity 1 day after administration that persisted for 2–3 additional days. These animals were completely protected from clinical disease, had no detectable virus replication as determined by the absence of viremia or virus in nasal secretions, were 3ABC and rRT-PCR negative, and had no detectable FMDV-specific neutralizing antibodies (Tables 3 and 7).

Clinical response of swine pretreated with Adt-pIFNβ and challenged by ID inoculation with FMDV A24

Groups of 3 swine were inoculated with medium (1×10^{10} FFU) or high doses (1×10^{11} FFU) of Adt-pIFNβ or with 1×10^{11} FFU of AdNull control vector and challenged by ID inoculation with FMDV A24 one day later (Table 4). The control group developed viremia by 2 dpc and clinical disease by 3 dpc. Two of 3 animals in the group inoculated with the medium dose of Adt-pIFNβ developed viremia by 4 or 5 dpc and clinical disease by 4 and 6 dpc, whereas the remaining animal never developed viremia or clinical disease, were 3ABC and rRT-PCR negative, and had a low neutralizing antibody response (Tables 4 and 7). All animals in the Adt-pIFNβ high-dose group were completely protected from clinical disease, had no viremia or virus in nasal swabs, and were 3ABC and rRT-PCR negative (Tables 4 and 7).

Clinical response of swine pretreated with Adt-pIFNα at one or more sites in the hind limb or neck and challenged by ID inoculation with FMDV A24

To possibly enhance the efficacy of pIFNα pretreatment, we examined 2 different anatomic delivery locations (hind limb versus neck) and also compared single versus multiple sites of IM inoculation in both locations (Table 5). In this

Table 3. Efficacy of Adenovirus Type 5 Vector Containing a Porcine Interferon α Gene in a Foot-and-Mouth Disease Virus A24 Cruzeiro Contact Challenge Experiment

Group (dose FFU)[a]	Animal no.	Antiviral act./pIFNα[b]	Viremia[c]	Shedding virus[d]	Clinical score[e]	PRN_{70}[f]	3ABC ELISA[g]
PBS (—)	9127	<25/72	$4/5\times10^3/2$	$5/9\times10^1/2$	5/11	1,600	P
	9128	<25/0	0	0	0	256	P
	9129	<25/0	$3/1\times10^3/2$	$4/6\times10^2/2$	3/17	1,600	P
AdNull (10^{11})	9130	25/11	0	$7/6\times10^1/2$	8/1	3,200	P
	9131	<25/0	$4/5\times10^3/2$	$4/4\times10^1/2$	5/15	3,200	P
	9132	<25/0	$4/9\times10^3/2$	$4/1\times10^3/4$	5/14	3,200	P
Adt-pIFNα (10^{10})	9133	800/4,376	0	0	0	<8	N
	9134	800/11,266	0	0	0	<8	N
	9135	400/2,530	$4/7\times10^3/2$	0	6/1	800	P
Adt-pIFNα (10^{11})	9136	800/13,788	0	0	0	<8	N
	9137	3,200/21,737	0	0	0	<8	N
	9138	3,200/40,305	0	0	0	<8	N

[a]Dose of inoculum per animal expressed as number of FFU in 2 mL of PBS.
[b]Antiviral activity (U/mL) of swine plasma in IBRS-2 cells and pIFNα (pg/mL) detected in swine plasma by ELISA at 1 day postinoculation.
[c]First day postchallenge (dpc) that viremia was detected, maximum amount of viremia in PFU/mL detected in sera samples, and the duration (days) of viremia.
[d]First dpc that shedding virus was detected, maximum amount of shedding virus in PFU/mL detected in nasal swab samples, and the duration (days) of shedding.
[e]dpc first signs of lesions/highest lesion score.
[f]Neutralizing antibody response reported as serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}) at 21 dpc.
[g]Detection of NS proteins in 21 dpc serum samples by 3ABC ELISA; N, negative; P, positive.

study we used a medium dose of 1×10^{10} FFU Adt-pIFNα, since in previous experiments this dose only protected some animals in the group or delayed the onset and severity of clinical disease in the remaining animals. The AdNull control-inoculated animals developed viremia by 1–2 dpc and clinical disease by 2 days (Table 5). All 4 groups administered a total of 1×10^{10} FFU Adt-pIFNα per animal had similar levels of antiviral activity and pIFNα protein detectable for 1–2 days. Two animals, 1 in the group given 4 shots of vector in the neck, no. 817, and the other in the group given 4 shots of vector in the hind limbs, no. 818, were found dead immediately before the start of the experiment or at 1 dpc, respectively. Postmortem histopathology analysis indicated that death was unrelated to the effects of administration of either Adt-pIFNα or FMDV infection (data not shown). The group of animals that received 4 shots in the neck showed the highest level of protection (Table 5). Both remaining animals in this group were completely protected from clinical disease, viremia, and virus in nasal swabs, and were 3ABC and rRT-PCR negative (Tables 5 and 7). In the group given 1 shot in the neck 1 animal was completely protected. The remaining 2 animals in this group both developed

Table 4. Serological and Clinical Response to Pretreatment of Swine with Adenovirus Type 5 Vector Containing a Porcine Interferon β Gene and Intradermal Challenge with Foot-and-Mouth Disease Virus A24 Cruzeiro

Group (dose FFU)[a]	Animal no.	Antiviral activity[b]	Viremia[c]	Shedding virus[d]	Clinical score[e]	PRN_{70}[f]	3ABC ELISA[g]
AdNull (10^{11})	44	<25	$2/7\times10^6/2$	$4/8\times10^0/1$	3/17	6,400	P
	45	<25	$2/5\times10^4/5$	$4/5\times10^2/3$	3/16	3,200	P
	46	<25	$2/2\times10^6/3$	$4/7\times10^2/2$	3/17	6,400	P
Adt-pIFNβ (10^{10})	47	62	$4/2\times10^3/2$	$4/8\times10^1/2$	6/15	6,400	P
	48	87	0	0	0	32	N
	49	43	$5/2\times10^3/1$	$5/4\times10^2/1$	4/15	3,200	P
Adt-pIFNβ (10^{11})	50	404	0	0	0	64	N
	51	429	0	0	0	64	N
	52	218	0	0	0	512	N

[a]Dose of inoculum per animal expressed as number of FFU in 2 mL of PBS.
[b]Antiviral activity (U/mL) of swine plasma in IBRS-2 cells.
[c]First day postchallenge (dpc) that viremia was detected, maximum amount of viremia in PFU/mL detected in sera samples, and the duration (days) of viremia.
[d]First dpc that shedding virus was detected, maximum amount of shedding virus in PFU/mL detected in nasal swab samples, and the duration (days) of shedding.
[e]dpc first signs of lesions/highest lesion score.
[f]Neutralizing antibody response reported as serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}) at 21 dpc.
[g]Detection of NS proteins in 21 dpc serum samples by 3ABC ELISA; N, negative; P, positive.

Table 5. Efficacy of Single-Site Versus Multiple-Site Inoculation of Adenovirus Type 5 Vector Containing a Porcine Interferon α Gene Against Intradermal Challenge with Foot-and-Mouth Disease Virus A24 Cruzeiro

Group[a]	*No. of shots*	*Inoculation site*	*Antiviral Act./ pIFNα*[b]	*Viremia*[c]	*Shedding virus*[d]	*Clinical score*[e]	*PRN_{70}*[f]	*3ABC ELISA*[g]
AdNull								
807	1	Neck	<25/183	$1/3\times10^5/4$	$3/3\times10^3/3$	2/17	8,000	P
808	1	Limb	<25/162	$2/2\times10^5/3$	$3/5\times10^3/3$	2/15	4,000	P
809	4	Neck	<25/168	$1/5\times10^5/4$	$3/4\times10^3/3$	2/17	4,000	P
810	4	Limb	<25/171	$2/1\times10^5/3$	$3/6\times10^3/3$	2/13	4,000	P
Adt-pIFNα								
805	1	Neck	200/3,455	$6/5\times10^1/1$	$1/4\times10^2/1$	8/9	64,000	P
806	1	Neck	100/1,548	0	0	10/4	32,000	P
811	1	Neck	800/4,886	0	0	0	<8	N
Adt-pIFNα								
812	1	Limb	200/6,918	0	0	0	64	N
813	1	Limb	200/4,010	$4/2\times10^3/3$	$2/6\times10^2/2$	6/12	16,000	P
814	1	Limb	25/432	$3/4\times10^2/1$	$2/3\times10^1/2$	4/15	4,000	P
Adt-pIFNα								
815	4	Neck	200/3,225	0	0	0	256	N
816	4	Neck	400/5,178	0	0	0	128	N
817	4	Neck	1600/11,967	NA[h]	NA[h]	NA[h]	NA[h]	NA[h]
Adt-pIFNα								
818	4	Limb	200/3,525	NA[h]	NA[h]	NA[h]	NA[h]	NA[h]
819	4	Limb	800/7,364	0	0	10/5	32,000	P
820	4	Limb	200/2,051	$5/1\times10^4/3$	$2/8\times10^2/2$	5/15	8,000	P

[a]Total dose of inoculum per animal was 1×10^{10} FFU in 2 mL of PBS.
[b]Antiviral activity (U/mL) of swine plasma in IBRS-2 cells and pIFNα (pg/mL) detected in swine plasma by ELISA at 1 day postinoculation.
[c]First day postchallenge (dpc) that viremia was detected, maximum amount of viremia in PFU/mL detected in sera samples, and the duration (days) of viremia.
[d]First dpc that shedding virus was detected, maximum amount of shedding virus in PFU/mL detected in nasal swab samples, and the duration (days) of shedding.
[e]dpc first signs of lesions/highest lesion score.
[f]Neutralizing antibody response reported as serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}) at 21 dpc.
[g]Detection of NS proteins in 21 dpc serum samples by 3ABC ELISA; N, negative; P, positive.
[h]Not applicable (NA). Animals died during the experiment. Cause not related to FMD.

clinical disease, but it was delayed until 8 and 10 dpc and less severe than the controls. In the group given 4 shots in the hind limbs the 2 remaining animals developed delayed clinical disease at 5 and 10 dpc. One animal in the group given 1 shot of Adt-pIFNα in the hind limb never developed clinical disease, viremia, or virus in nasal swabs, and was 3ABC and rRT-PCR negative (Tables 5 and 7). The other 2 animals in this group developed lesions at 4 and 6 dpc, and viremia and virus in nasal swabs was detectable but was 10–1,000-fold lower than the controls.

Clinical response of swine pretreated with Adt-pIFNα and ID challenged with FMDV Asia-1

On the basis of the multiple shot experimental results, swine were inoculated IM with medium (1×10^{10} FFU) or high doses (1×10^{11} FFU) of Adt-pIFNα or AdNull (1×10^{11} FFU) at 4 sites in the neck and challenged with Asia-1 (Table 6). All control AdNull-inoculated animals developed viremia at 1 dpc and lesions at 2 dpc. All animals in the 1×10^{10} FFU Adt-pIFNα-inoculated group had detectable antiviral activity and pIFNα protein for 1–2 days. Two of 3 animals in this group were protected from clinical disease, did not develop viremia, and were 3ABC and rRT-PCR negative (Tables 6 and 7). The third animal in this group developed a low level of viremia (1,000–10,000-fold lower than any of the control animals) for 1 day, shed 10–80-fold less virus than the controls, and developed lesions at 5 dpc. All animals treated with 1×10^{11} FFU Adt-pIFNα had antiviral activity and pIFNα protein for 3–4 days and were completely protected from challenge (Tables 6 and 7).

Discussion

Previously, we demonstrated that swine inoculated IM at 1 site in the hind limb with an Ad5 vector containing the gene for pIFNα were protected from clinical disease and virus replication when challenged by ID inoculation 1 day after with FMDV A24 Cruzeiro (Chinsangaram and others 2003; Moraes and others 2003). In this study we have extended our previous work and examined the efficacy of type I IFN pretreatment of swine challenged with several FMDV serotypes by either ID inoculation or direct contact exposure. We showed that a dose of 10^{11} FFU Adt-pIFNα administered at 1 site in the hind limb could protect all swine challenged, 1 day postadministration, by ID inoculation with FMDV serotypes A24 Cruzeiro or O1 Manisa. Similarly, at this dose, Adt-pIFNβ completely protected swine against challenge with A24 Cruzeiro. In addition, animals pretreated with 10^{11} FFU Adt-pIFNα and challenged by direct contact exposure to A24 Cruzeiro–infected donor animals for 18 h were protected.

Table 6. Serological and Clinical Response of Swine Pretreated with Adenovirus Type 5 Vector Containing a Porcine Interferon α Gene and Intradermal Challenged with Foot-and-Mouth Disease Virus Asia-1

Group (dose FFU)[a]	*Animal no.*	*Antiviral act./pIFNα*[b]	*Viremia (pfu/mL)*[c]	*Shedding virus*[d]	*Clinical score*[e]	*PRN_{70}*[f]	*3ABC ELISA*[g]
AdNull (10^{11})	24094	<25/111	$1/4.7\times10^5/4$	$2/8.8\times10^2/2$	2/17	4,096	P
	24095	<25/192	$1/1.5\times10^5/3$	$3/4.3\times10^2/2$	2/15	1,024	P
	24097	<25/41	$1/1.6\times10^6/4$	$2/1.4\times10^2/2$	2/16	2,048	P
Adt-pIFNα (10^{10})	24092	100/1,163	0/0/0	0/0/0	0/0	32	N
	24093	100/2,024	0/0/0	0/0/0	0/0	16	N
	24096	25/908	$5/3.5\times10^2/1$	$5/1\times10^1/1$	5/9	512	P
Adt-pIFNα (10^{11})	24088	1,600/19,855	0/0/0	0/0/0	0/0	16	N
	24090	1,600/26,876	0/0/0	0/0/0	0/0	<8	N
	24091	1,600/17,505	0/0/0	0/0/0	0/0	<8	N

[a]Total dose of inoculum per animal expressed as number of FFU in 2 mL of PBS.
[b]Antiviral activity (U/mL) and pIFNα (pg/mL) at 1 day postinoculation.
[c]First day postchallenge (dpc) that viremia was detected, maximum amount of viremia in PFU/mL detected in sera samples, and the duration (days) of viremia.
[d]First dpc that shedding virus was detected, maximum amount of shedding virus in PFU/mL detected in nasal swab samples, and the duration (days) of shedding.
[e]dpc first signs of lesions/highest lesion score.
[f]Neutralizing antibody response reported as serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}) at 21 dpc.
[g]Detection of NS proteins in 21 dpc serum samples by 3ABC ELISA; N, negative; P, positive.

We were also able to considerably enhance the potency of Adt-pIFNα by administration of this vector at 4 sites in the neck compared with 1 site in the hind limb so that a 10-fold lower dose could completely protect 4 of 5 animals against ID challenge with either A24 Cruzeiro or Asia-1.

In the current study we used the same Adt-pIFNα and Adt-pIFNβ vector production lots in all of our experiments. Therefore, we initially performed a dose–response experiment with these vectors to determine the optimal doses needed to induce levels of pIFNα and/or antiviral activity that in previous experiments, with vector produced in our laboratory, were required to induce protection against clinical disease. We found that doses of 10^{10} or 10^{11} FFU-induced levels of pIFNα and antiviral activity that we predicted

Table 7. Adenovirus Type 5 Vector Containing a Porcine Interferon α/β Gene Efficacy Studies in Swine: Evaluation of Sterile Protection in Clinically Protected Swine

Study	*Animal no.*	*IFN dose (FFU)*	*Viremia*	*Shedding*	*PRN_{70}*[a]	*3ABC*[b]	*rRT-PCR*[c]
IFNα, A24 challenge, Table 2	20962	10^{11}	0	0	512	N	N
	20963	10^{11}	0	0	<8	N	N
	20964	10^{11}	0	0	512	N	N
IFNα, O1M challenge, Table 2	959	10^{10}	0	0	8	N	N
	961	10^{11}	0	0	<8	N	N
	962	10^{11}	0	0	<8	N	N
	963	10^{11}	0	0	<8	N	N
IFNα, A24 contact challenge, Table 3	9133	10^{10}	0	0	<8	N	N
	9134	10^{10}	0	0	<8	N	N
	9136	10^{11}	0	0	<8	N	N
	9137	10^{11}	0	0	<8	N	N
	9138	10^{11}	0	0	<8	N	N
IFNβ, A24 challenge, Table 4	48	10^{10}	0	0	32	N	N
	50	10^{11}	0	0	64	N	N
	51	10^{11}	0	0	64	N	N
	52	10^{11}	0	0	512	N	N
IFNα, A24 challenge, Table 5	811	10^{10}	0	0	<8	N	N
	812	10^{10}	0	0	64	N	N
	815	10^{10}	0	0	256	N	N
	816	10^{10}	0	0	128	N	N
IFNα, Asia-1 challenge, Table 6	24092	10^{10}	0	0	32	N	N
	24093	10^{10}	0	0	16	N	N
	24088	10^{11}	0	0	16	N	N
	24090	10^{11}	0	0	<8	N	N
	24091	10^{11}	0	0	<8	N	N

[a]Neutralizing antibody response reported as serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}) at 21 days postchallenge (dpc).
[b]Detection of NS proteins in 21 dpc serum samples by 3ABC ELISA; N, negative; P, positive.
[c]rRT-PCR of serum samples at 1–7 dpc. N = Ct ≥40; P = Ct <40.
rRT-PCR, real-time reverse transcriptase–polymerase chain reaction.

would partially or completely protect swine when challenged 1 day postadministration. Further, we used the same lot of each swine-derived FMDV serotype in all challenge experiments and determined that the dose of each serotype required to reliably induce clinical disease in all control animals by 2–3 days postchallenge was 10^5 $TCID_{50}$. While this dose is 10-fold higher than that recommended by the OIE, we preferred to use a relatively severe challenge to test our model.

The most common mechanism of spread of FMD to swine is by direct contact exposure with infected animals (Alexandersen and others 2003). Therefore, to examine the efficacy of rapid protection against natural infection of swine pretreated with Adt-pIFNα, we utilized an FMD contact transmission model (Pacheco and others, manuscript in preparation). All donor animals developed lesions by 2 dpc and all recipient animals had detectable virus in nasal swab samples 1 day postcontact challenge, indicating a successful contact exposure with the donor animals. Five of the 6 control-inoculated recipient animals (PBS and AdNull) developed clinical disease and all of these animals, including the animal that did not develop lesions, had a high FMDV-specific neutralizing antibody response and were 3ABC ELISA positive, demonstrating that FMDV replication occurred. We found that all animals pretreated with 10^{11} FFU Adt-pIFNα and 2 of 3 animals pretreated with 10^{10} FFU were completely protected from contact challenge. These results confirm that Adt-pIFNα can rapidly protect swine not only against FMDV using an OIE-approved ID challenge model, even at a 10-fold higher challenge dose than recommended, but also against challenge by the natural route of infection. Moreover, since we obtained better protection with less Adt-pIFNα in the contact challenge experiment compared with ID challenge, this suggests that in a natural FMD outbreak the lower dose may be sufficient to limit disease spread beyond the initial farm on which an outbreak occurs.

Although all animals inoculated with 10^{11} FFU Adt-pIFNα/β were protected from FMDV challenge, they developed transient jaundice and generally did not eat well (data not shown; animals inoculated with this dose of the control vectors did not develop jaundice or other adverse effects). Recovery required 2–3 days. To avoid these side effects as well as develop a more potent biotherapeutic, we attempted to lower the protective dose of Adt-pIFNα by comparing single-site versus multiple-site IM inoculation in the hind limb and neck. The data clearly show that inoculation at 4 sites in the neck with 10^{10} FFU Adt-pIFNα was the most efficacious method of administration since both animals in this group were completely protected, and based on the antiviral activity detected in the animal in this group that died 1 dpc, we predict that this animal would also have been protected (Table 5). Further, 2 of the 3 animals administered 10^{10} FFU Adt-pIFNα by this method in the Asia-1 challenge experiment were also completely protected (Tables 6 and 7).

There are limited studies comparing the efficacy of single-site versus multiple-site inoculation, and most of these are vaccination studies. Gardiner and others (2006) showed that multiple-site inoculation of mice at different anatomic locations with a DNA vector containing HIV Gag-specific peptides improved both cellular and humoral immune responses compared with inoculation with the same dose at only 1 site. The authors attributed the enhanced responses to multiple factors, including antigen loading of antigen presenting cells and presentation/recruitment of antigen-specific naïve T-cells at the regional lymph nodes. Similarly, Wansley and others (2008) found that mice vaccinated at multiple sites with a tumor antigen have an enhanced antigen-specific T cell response and resulted in lower tumor volume compared with mice vaccinated at a single site. In the current study the only parameters that we examined in addition to clinical score were the presence of pIFNα protein and antiviral activity in plasma. In both assays the highest level of protein and antiviral activity was detected in animals inoculated at 4 sites in either the hind limb or neck. However, these quantitative differences were not statistically significant when these 2 groups were compared with the other groups. Perhaps based on our previous studies (Diaz-San Segundo and others 2010), examination of ISGs in specific tissues and/or examination of the number and maturation status of DCs and NK cells might show a relationship that would help explain the improved efficacy of the multiple-site inoculation approach.

We examined a number of criteria to assess whether IFN pretreatment completely blocked productive FMDV replication in animals that had no detectable clinical disease, that is, induced sterile immunity (Table 7). On the basis of the absence of (1) viremia, (2) virus shedding, (3) viral RNA in serum by rRT-PCR, (4) antibodies against viral NS protein 3ABC, and (5) the absence or very low levels of FMDV-specific neutralizing antibodies (PRN_{70} less than 100), pretreatment induced sterile immunity in 20 of 25 animals that had no clinical disease. The 5 remaining animals only had a significant FMDV-specific neutralizing antibody responses, that is, nos. 20962, 20964, 52, 815, and 816. The data for these 5 animals suggest that there was very limited virus replication. Nevertheless, IFN pretreatment clearly either dramatically reduced or completely inhibited productive FMDV replication, thereby significantly limiting virus shedding into the environment.

In subsequent studies we plan to examine alternate routes of delivery of the Adt-IFNs. We will also examine if administration of IFNs in combination with other molecules capable of inducing an innate immune response might result in a more robust response and potentially lower the effective Adt-IFN doses needed to rapidly and sterilely protect swine and other susceptible species from FMD.

Acknowledgments

This research was supported in part by the Plum Island Animal Disease Research Participation Program administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Department of Agriculture (appointment of Camila C.A. Dias and Fayna Diaz-San Segundo); by CRIS project no. 1940-32000-053-00D, Agricultural Research Service (ARS), U.S. Department of Agriculture (M. J. Grubman, T. de los Santos); and by reimbursable agreement no. 60-1940-7-47 with the Department of Homeland Security (M.J. Grubman).

We thank Juan Pacheco and Luis L. Rodriguez ARS, Plum Island Animal Disease Center (PIADC), for FMDV stocks; George Smoliga and Ethan Hartwig ARS, PIADC, for help with rRT-PCR analysis; and Douglas E. Brough and Damodar Ettyreddy from GenVec, Inc., for supplies of the Adt vectors. We also thank Karen Moran, Ian Olesen, and Samia

Metwally of the Foreign Animal Disease Diagnostic Laboratory (FADDL) at PIADC for performing the 3ABC ELISA assays; Fawzi Mohamed, FADDL, for performing histopathological analysis on the animals that died in the various trials; and the animal care staff at PIADC for their professional support and assistance. We gratefully acknowledge Drs. David Brake and Eva Perez for critical reading of the manuscript.

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Dr. Marvin J. Grubman
Plum Island Animal Disease Center
Agricultural Research Service
U.S. Department of Agriculture
North Atlantic Area, P.O. Box 848
Greenport, NY 11944

E-mail: marvin.grubman@ars.usda.gov

Received 27 May 2010/Accepted 20 June 2010